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AUTOMATIC ANALYSIS OF SERUM LACTATE DEHYDROGENASE ISOENZYMES BY HIGH-PERFORMANCE ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The repetitive analysis of serum lactate dehydrogenase (LDH) isoenzymes has been performed on a weak anion exchanger (TSKgel DEAE-5PW), which was developed by introducing diethylaminoethyl groups into TSKgel G5000PW (10 μm particle diameter) — a hydrophilic polymer-based material of large pore size — for high-performance gel chromatography. By use of this anion exchanger, a high-pH (> 8.0) solvent could be used and the albumin peak was completely separate from the LDH isoenzyme peaks. After 10 successive analyses with an autosampler, the coefficient of variation of the LDH isoenzyme elution times was $\leq$ 0.90%, and the coefficient of variation for peak areas was $\leq$ 3.85%. After 40 successive analyses, resolution between isoenzymes was generally > 1.25. This column can be used for more than 300 intermittent injections of human serum.

INTRODUCTION

Recently, in many laboratories, the activities of lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase; EC 1.1.1.27) isoenzymes in serum have been determined by use of electrophoresis, but this method has several problems: the procedure is too complicated, it requires long analysis times and the activities of unstable components, i.e. LDH-3, LDH-4 and LDH-5, are apt to be lost in measurement [1—3]. Using high-performance liquid chromatography (HPLC), the loss of these activities is very small because of the short analysis time under mild conditions, and HPLC is capable of a fully automatic analysis. The results of determinations of human serum LDH isoenzymes by HPLC have been reported in large numbers over the past few years [4—7], but

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they resulted in analysis of only one sample, and there was no report on repetitive analysis with an autosampler.

In this paper, the repetitive analysis of LDH isoenzymes was carried out on a column packed with a weak anion exchanger of hydrophilic polymer-based material [8]. Linear gradient elution with sodium chloride was used to determine the analysis time and the stability of the baseline after separation was complete. Successive analysis could be performed with a 30-min cycle per sample.

EXPERIMENTAL

Apparatus

HPLC was performed on an SP8700 solvent delivery system and on SP8750 organizer (Spectra-Physics, San Jose, CA, U.S.A.). Samples were applied with an AS-48 autosampler (Toyo Soda, Tokyo, Japan) equipped with a 100-μl loop. For fluorometric detection, an RF-500LC (Shimadzu, Kyoto, Japan) was used with both a C-R1A (Shimadzu) and a YEW Type 3066 pen recorder (Yokogawa Electric Works, Tokyo, Japan). Reaction reagent was carried away by a Technicon autoanalyzer proportioning pump (Technicon Instruments, Tarrytown, NY, U.S.A.). Reaction solution was incubated with Thermobox Model M-3 (Thermonics, Tokyo, Japan). The pH was adjusted by an M 83 Autocal pH meter (Radiometer, Copenhagen, Denmark).

Materials

Chromatograms were run on TSKgel DEAE-5PW (10 μm, 75 × 7.5 mm I.D.) (Toyo Soda). Tris(hydroxymethyl)aminomethane and lithium L(+)-lactate were purchased from Sigma (St. Louis, MO, U.S.A.). Hydrochloric acid and sodium chloride were from Wako (Osaka, Japan). β-NAD^+ (nicotinamide-adenine dinucleotide, oxidized) was from Oriental Yeast (Osaka, Japan). Brij-35 (30% solution) was purchased from Technicon International Chemicals Co. (Saint Denis, France).

Solvents A and B were prepared as follows: solvent A: tris(hydroxymethyl)-aminomethane (2.4228 g) was made up to 2 l with distilled water and a small amount of 1 *M* hydrochloric acid to obtain 10 m*M* Tris—HCl buffer (pH 8.0) at 25°C; solvent B: 58.45 g of sodium chloride was dissolved in 2 l of solvent A to obtain 0.5 *M* sodium chloride in 10 m*M* Tris—HCl buffer (pH 8.0). Tris-aminomethane (48.456 g) was made up to 1 l with distilled water and a small amount of 6 *M* hydrochloric acid to obtain 400 m*M* Tris—HCl buffer (pH 8.7). Reaction reagent was prepared by dissolving 6.72 g of lithium L(+)-lactate and 2.868 g of β-NAD^+ in 1 l of 400 m*M* Tris—HCl buffer (pH 8.7) to obtain 70 m*M* L(+)-lactate and 4 m*M* β-NAD^+ in buffer, mixed with 3.3 ml of Brij-35 (30% solution) before use. All reagents were commercially obtained, either reagent grade or the purest grade obtainable.

Methods

Elution conditions were as follows: solvent A, 10 m*M* Tris—HCl buffer (pH 8.0); solvent B, 0.5 *M* sodium chloride in solvent A; starting eluent, 7% solvent B for 2 min. Linear gradients: 7—22% solvent B in 3 min, 22—35% solvent B

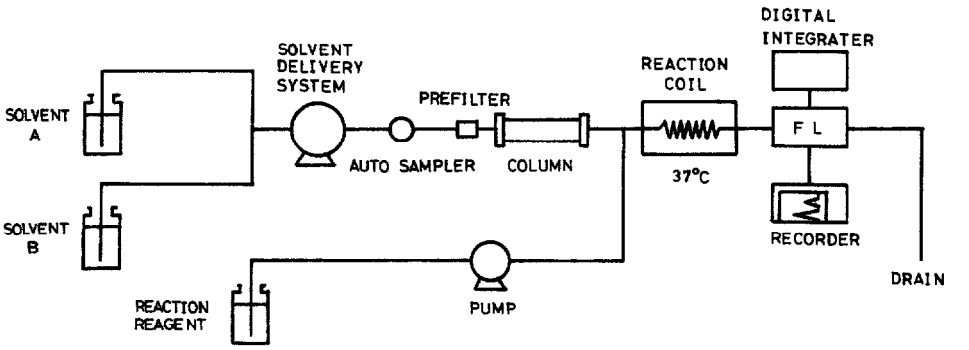


Fig. 1. High-performance liquid chromatographic system for the determination of LDH isoenzymes.

in 16 min and 35—80% solvent B in 1 min. Elute with 80% solvent B for 1 min, and with starting eluent for 7 min. The next sample can then be injected.

For the reaction reagents, 400 m*M* Tris—HCl buffer (pH 8.7) containing 70 m*M* L(+)-lactate, 4 m*M* β-NAD^+ and 0.1% Brij-35 were used [9]. The effluent was mixed with this reagent, and incubated for 3 min at 37°C; NADH (nicotinamide-adenine dinucleotide, reduced) formed in this reaction was quantified fluorometrically at λ_{ex} = 370 nm and λ_{em} = 465 nm (Fig. 1).

RESULTS AND DISCUSSION

One of the chromatograms that were obtained by use of this gradient-elution method during the 40 successive injections of four-fold diluted human serum with solvent A is shown in Fig. 2. LDH-5, LDH-4, LDH-3, LDH-2 and LDH-1

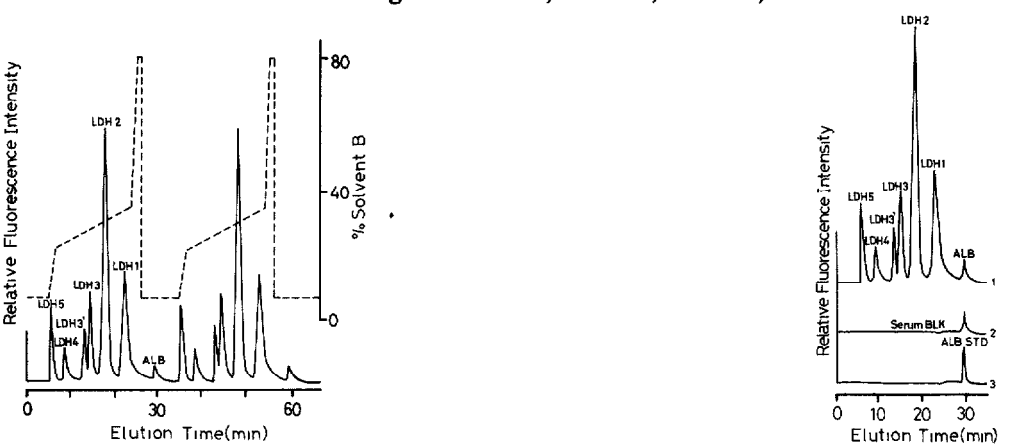


Fig. 2. Chromatogram of LDH isoenzymes obtained by high-performance ion-exchange chromatography on TSKgel DEAE-5PW. Chromatographic conditions: solvent A, 0.01 *M* Tris—HCl buffer (pH 8.0); solvent B, 0.5 *M* sodium chloride in solvent A; sample, four-fold diluted human serum with solvent A; sample volume, 100 μl; flow-rate, 1.0 ml/min. ALB = albumin.

Fig. 3. Elution profile of LDH isoenzymes and human albumin on TSKgel DEAE-5PW. Samples: (1) human serum, (2) human serum (minus blank); (3) human albumin (5 g/dl). ALB STD = albumin standard; Serum BLK = serum blank.

were eluted in this order, which is commonly denoted in electrophoretic literature. Further, multiplicity was observed in LDH-3: named LDH-3′ (first peak) and LDH-3 (second peak), respectively [10]. A non-LDH fluorescence peak eluted after LDH-1 was determined on albumin (Fig. 3).

The within-run reproducibilities for the peak areas and elution times of isoenzymes were investigated by making ten successive injections of human serum. Consequently, the coefficients of variation (C.V.) for elution times were LDH-1 = 0.50%, LDH-2 = 0.55%, LDH-3 = 0.56%, LDH-4 = 0.90%, LDH-5 = 0.83% and those for peak areas were LDH-1 = 3.57%, LDH-2 = 2.25%, LDH-3 = 0.56%, LDH-4 = 3.85%, LHD-5 = 2.06% (Table I). LDH-3, however, gave two peaks, so elution times were evaluated for the second peak and peak area was the sum of the first and second peak.

TABLE I

WITHIN-RUN REPRODUCIBILITY OF ELUTION TIME AND PEAK AREA OF LDH ISOENZYMES

Total activity = 480 I.U./l; n = 10.

LDH isoenzyme	Elution time (mean ± S.D.) (min)	Coefficient of variation (%)	Peak area (mean ± S.D.) (I.U./l)	Coefficient of variation (%)
LDH-1	26.12 ± 0.13	0.50	127 ± 4.54	3.57
LDH-2	19.93 ± 0.11	0.55	190 ± 4.27	2.25
LDH-3	16.05 ± 0.09	0.56	93 ± 1.72	1.85
LDH-4	10.12 ± 0.09	0.90	34 ± 1.31	3.85
LDH-5	6.00 ± 0.05	0.83	36 ± 0.74	2.06

The limit of sensitivity was ca. 0.9 I.U./l, and the linear dynamic range was 20—1200 I.U./l (Fig. 4).

Resolution and elution times were investigated during the 40 successive injections of diluted human serum with solvent A. As a result, elution times had a C.V. of < 2%, and for resolution (R_s) LDH 1—2: $1.99 < R_s < 2.01$, LDH 2—3: $1.25 < R_s < 1.28$, LDH 3—4: $1.95 < R_s < 1.97$, LDH 4—5: $1.54 < R_s < 1.58$ (Fig. 5).

The lifetime of TSKgel DEAE-5PW was investigated by making more than 300 intermittent injections of human serum. Two chromatograms, from the 1st and the 300th run of this column separation, are shown in Fig. 6. The end of column life was not reached in these tests, as the resolution of the LDH isoenzymes was maintained after more than 300 intermittent injections [11].

There is a wide difference in isoelectric point (pI) between LDH-3, LDH-4 and LDH-5; also, the pI of LDH-1, LDH-2 and LDH-3 were very close together. Therefore, the long analysis time is due to the wide distance between the peak of LDH-3, LDH-4 and LDH-5 if a gentle, single, linear gradient method with sodium chloride is carried out. On the other hand, if a steep, single, linear gradient method is used, the resolution of LDH-1, LDH-2 and LDH-3 is reduced. For the purpose of superior resolution of five components in a short time, it is necessary that several varied gradient-elution methods are combined.

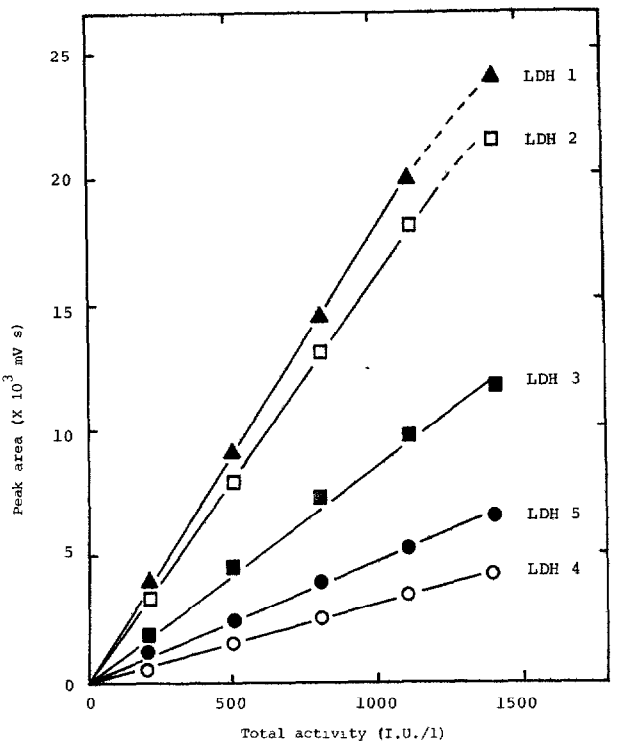


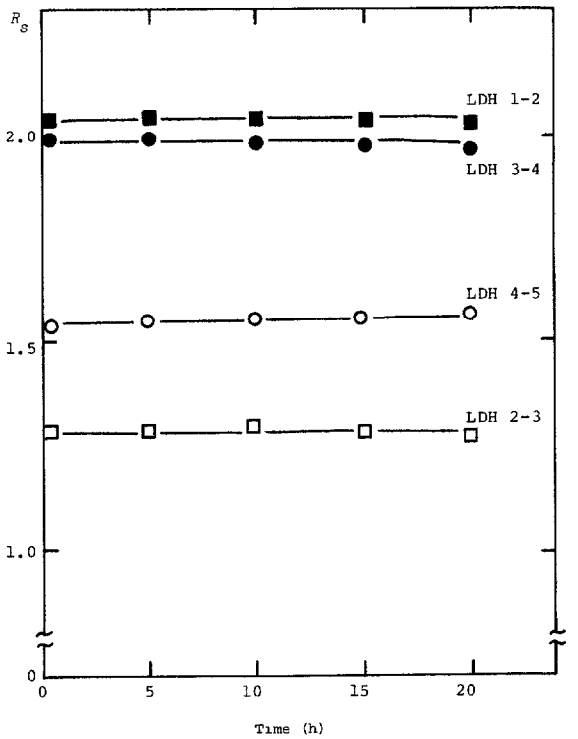
Fig. 4. Linearity between total LDH activity and peak area. Sample: diluted human serum of total activity 1420 I.U./l with solvent A.

Compared with the widely used electrophoretic method for determining the activity of LDH isoenzymes, HPLC in this study provides excellent resolution, a fine within-run reproducibility in peak areas, the cost per sample is halved, and successive analysis is completely automatic.

There are several ways of shortening analysis times: scaling down the column size, increasing the flow-rate, shortening the reaction time, etc. Accordingly, as the reaction time is shortened to less than the 3 min used in this study, the sensitivity of detection should be increased. But there are possibilities that baseline noise is increased on the chromatogram because the sensitivity of the detector must be increased to a maximum, owing to the presence of a little NADH, if a sample with low total LDH activity (< 100 I.U./l) is to be analysed using a shorter reaction time than 3 min. Therefore, it seems necessary that 3 min should be the minimum for the reaction, so as not to confuse small peaks with baseline noise.

In this research, the successive analyses were carried out on the column (75 × 7.5 mm I.D.) at a flow-rate of 1.0 ml/min using the 30-min cycle per sample, and the excellent durability of the column was proved. Currently, it is under investigation as to what degree the column size can be shortened and the flow-rate increased for the repetitive analysis, in keeping with the good resolution and durability.

HPLC in this report gave a much superior resolution, lower cost per sample, and a fully automatic analysis compared with electrophoresis. A slight change



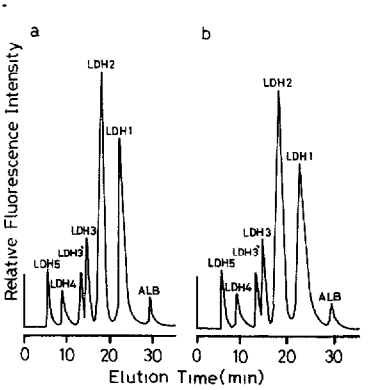


Fig. 5. Changes in resolution for the peak of LDH isoenzymes during 40 successive injections on TSKgel DEAE-5PW.

Fig. 6. Chromatograms from the 1st (a) and the 300th (b) run of TSKgel DEAE-5PW separation.

in behaviour of the multiplicities of isoenzymes could be observed on the chromatograms. The lifetime of TSKgel DEAE-5PW was much longer than that of the usual column. In summary, this method could be usefully introduced into the routine testing of serum LDH isoenzymes.

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